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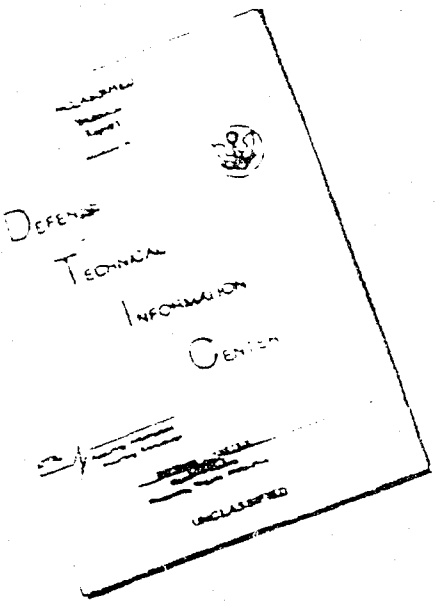

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TECHNICAL MANUSCRIPT 42

THE PATHOGENESIS OF PLAGUE. I. A STUDY OF THE CORRELATION BETWEEN VIRULENCE AND RELATIVE PHAGOCYTOSIS RESISTANCE OF SOME STRAINS OF *Pasteurella pestis*

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THE PATHOGENESIS OF PLAGUE
I. A STUDY OF THE CORRELATION BETWEEN VIRULENCE AND RELATIVE PHAGOCYTOSIS RESISTANCE OF SOME STRAINS OF PASTEURELLA PESTIS

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ABSTRACT

This report presents evidence that when avirulent strains of P. pestis that produce the capsular antigen Fraction I are grown under conditions that induce a high degree of phagocytosis resistance in typical virulent strains, they also acquire the same degree of resistance. Virulent strains that do not produce detectable amounts of this antigen acquire a much more limited degree of resistance to phagocytosis; in fact, they are not at all resistant to ingestion by guinea pig macrophages. It is suggested that the major factors determining the virulence of P. pestis are not associated with resistance to phagocytosis, but rather, with the intracellular survival and multiplication of the bacilli following phagocytosis.

I. INTRODUCTION

The ability of the plague bacillus to become resistance to phagocytosis has long been regarded as a major determinant of its virulence. Meyer[1] and Englesberg et al[2] correlated the virulence of Pasteurella pestis with the ability to synthesize an envelope or capsular substance, called Fraction I by Baker et al,[3] that protects the bacterium from phagocytosis. Burrows and Bacon attribute the virulence of P. pestis to its ability to synthesize two other antigens, designated V and W, which apparently prevent phagocytosis of a virulent strain incapable of producing Fraction I.[4,5]

In a previous publication by Janssen et al,[6] we presented circumstantial evidence that virulent P. pestis organisms that were highly resistant to phagocytosis by neutrophiles and free macrophages were rapidly removed from the blood by the fixed macrophages of the reticuloendothelial system (RES). Subsequent histopathologic studies in our laboratory have revealed that fixed macrophages readily ingest these so-called "phagocytosis-resistant" virulent organisms.* The possibility that the virulence of P. pestis may also reside in its ability to survive and multiply within fixed macrophages, or in its ability to multiply at a rate exceeding the capacity of the RES to destroy its progeny, was suggested. Cavanaugh and Randall[7] showed that virulent P. pestis survived and grew within free macrophages. These and other observations have led us to conclude that, as a rule, all of the phagocytic cells of the nonimmune host animal are ineffective against virulent P. pestis, and therefore resistance to phagocytosis plays a minor if not insignificant role in determining the virulence of this pathogen. The arguments in favor of this conclusion will be developed in forthcoming reports from these laboratories, of which this is the first.

Evidence in this report demonstrates that avirulent strains producing the capsular antigen Fraction I, but no V and W antigens, are more highly resistant to ingestion by free phagocytes than noncapsulated virulent strains producing the "virulence" antigens V and W. The implication of these observations is discussed.

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* Unpublished observations.

II. METHODS

A. BACTERIAL STRAINS

Two typical virulent strains (producing Fraction I, and V and W antigens) were employed. These are designated the Alexander and Joe strains. Two atypical virulent strains called M-23 and M-41 (producing V and W, but no visible capsulation), as well as the avirulent strains A-12, A-1224, A-4, and AD-5 (producing capsular antigen Fraction I, but no V and W) were also used in these studies.

B. CULTURAL CONDITIONS

Phagocytosis-susceptible cells were readily obtained by growing any of the strains in Difco heart infusion broth (HIB) containing 0.06 per cent $MgSO_4 \cdot 7H_2O$ and one per cent xylose at 26°C for 24 hours on a reciprocating shaker. Phagocytosis resistance of typical virulent strains could be induced by growing them in HIB containing five per cent heparinized guinea pig blood at 37°C for 24 hours on a drum rack rotating at 30 rpm, or by injecting 5×10^8 organisms contained in 25 milliliters of HIB into the peritoneal cavity of a guinea pig and harvesting the heparinized exudate 18 hours later.* All of the strains were grown under conditions known to induce phagocytosis resistance in typical virulent strains; however, when the avirulent strains were injected into the peritoneal cavities of guinea pigs, the resulting exudate cultures usually contained relatively few organisms and quickly clotted. Strain A-1224 occasionally produced a usable peritoneal exudate culture when 5×10^8 organisms in heparinized HIB were injected. Sodium heparin (0.1 milligram per milliliter) was the anticoagulant employed. All exudate cultures were centrifuged at 500 rpm for five minutes to remove leucocytes.

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* In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

C. IN VITRO ASSAY OF RELATIVE PHAGOCYTOSIS RESISTANCE

Details of the standard procedure used in this assay were presented in a previous communication.[6] In brief, the total number of bacteria per milliliter of each culture was determined in a Petroff-Hauser counting chamber, and the concentration of bacteria was adjusted by low-speed centrifugation so that roughly the same number would be available to phagocytes in each test system. The test system consisted of 0.9 milliliter of a leucocyte suspension and 0.1 milliliter of culture in a 100-mm by 12.5-mm serological test tube. The mixtures of bacteria and leucocytes were placed on a drum rack rotating at 1/5 rpm* and incubated for 30 minutes at 38.8°C (the normal rectal temperature of guinea pigs). Smear samples were then taken, air-dried, fixed for ten minutes in methyl alcohol, and stained with Giemsa's blood stain. The total number of P. pestis organisms contained in 100 neutrophiles and 100 macrophages, as well as the percentage of each of these phagocytic cell types containing P. pestis, were determined.

D. SOURCE OF LEUCOCYTES

Leucocytes were obtained from mice by bleeding 20 Swiss-Webster mice from the heart and centrifuging the heparinized blood for 15 minutes at 2000 rpm; then, the plasma and buffy coat from ten milliliters of blood were removed and mixed in order to resuspend the leucocytes. Leucocytes were obtained from guinea pigs (Hartley strain) by injecting 25 milliliters of sterile 7.6 per cent sodium caseinate solution intraperitoneally into an animal and harvesting the resulting exudate 24 hours later. Heparin was added immediately. In every case, the total number of leucocytes per milliliter of the suspensions was determined.

E. ANTIGEN ANALYSIS

Cultures were analyzed specifically for Fraction I, V and W antigens using a gel diffusion technique described previously.[9]

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* Experience had shown that this slow rotation favored phagocytosis of encapsulated organisms, probably through the phenomenon of surface phagocytosis described by Smith and Wood.[8]

III. RESULTS

The initial experiments, using neutrophiles from mice, were designed to determine whether avirulent strains grown under conditions known to induce phagocytosis resistance in a typical virulent strain would also become resistant. These strains resembled the virulent Alexander strain when grown in HIB containing five per cent guinea pig blood at 37°C in that they produced the capsular antigen Fraction I and became highly resistant to phagocytosis but unlike the virulent strain, they did not produce V and W antigens. Also, in contrast to the virulent Alexander and M-23 strains, the avirulent strains did not grow sufficiently well in the peritoneal cavity of guinea pigs to permit the use of this cultural condition for induction of phagocytosis resistance. The virulent noncapsulated strain M-23 resembled the typical virulent strain Alexander after growth in the guinea pig peritoneal cavity in that it produced V and W antigens but it did not produce Fraction I antigen, and acquired only a slight degree of resistance to phagocytosis by neutrophiles from mice. Four separate experiments were performed with essentially identical results. The results of one such experiment are recorded in Table I.

In the second series of experiments, neutrophiles and free macrophages from both mice and guinea pigs were used, and the P. pestis strains were grown exclusively in the peritoneal cavities of guinea pigs in order to assure maximal induction of phagocytosis resistance. It was possible to obtain a peritoneal exudate culture of the encapsulated avirulent strain A-1224 in an occasional guinea pig by increasing the inoculum size to 5×10^9, and by adding 0.1 milligram per milliliter of sodium heparin to the inoculum. Avirulent strains usually disappeared rapidly from the peritoneal cavity of guinea pigs, and any resulting exudate had a marked tendency to clot before it could be harvested; therefore, it was impossible to test cultures of most avirulent strains after growth under these conditions. Three separate experiments were performed with very similar results. A representative experiment is recorded in Table II. It may be seen that the typical virulent strains Alexander and Joe produced the antigens Fraction I, V and W, and became highly resistant to ingestion by free phagocytes from both mice and guinea pigs. The encapsulated avirulent strain A-1224 also produced Fraction I antigen and became highly resistant to phagocytosis, but failed to produce detectable amounts of V and W antigens. Atypical virulent strains M-23 and M-41 both produced V and W antigens and acquired a fairly high degree of resistance to neutrophiles from both classes of animals. However, they did not produce detectable amounts of Fraction I, acquired only a moderate degree of resistance to free macrophages from mice, and were not resistant at all to phagocytosis by free macrophages from guinea pigs. It should be pointed out that the M-23 strain used in this second series of experiments had previously been subjected to five serial passages in guinea pigs, and its virulence for both mice and guinea pigs was of a considerably higher order than when the initial experiments were performed. All of the avirulent strains remained avirulent during the course of these studies.

TABLE I. A COMPARISON OF THE PHAGOCYTOSIS RESISTANCE OF SOME VIRULENT AND AVIRULENT STRAINS OF *PASTEURELLA PESTIS* TO MOUSE NEUTROPHILES[a]

TEST SYSTEM			RESULTS			
P. pestis Culture		*P. pestis* / Leucocyte	Neutrophile Phagocytic Activity[b]	Antigens Detected		
Strain	Cultivation			FI[e]	V	W
Virulent strains:						
Alexander	HIB,[c] 26°C	15.6	669/86	-	-	-
Alexander	HIB + 5% blood, 37°C	15.6	3/2	+	+	+
Alexander	*In vivo*[d] (38.8°C)	22	0/0	+	+	+
M-23	*In vivo* (38.8°C)	18.9	122/52	-	+	+
Avirulent strains:						
A-12	HIB + 5% blood, 37°C	14.7	8/4	+	-	-
A-4	HIB + 5% blood, 37°C	15.6	4/4	+	-	-
A-1224	HIB + 5% blood, 37°C	13.6	4/4	+	-	-
AD-5	HIB + 5% blood, 37°C	15.6	16/6	+	-	-
Control	(uninoculated HIB medium)	0	0/0	-	-	-

a. Contained in the blood.
b. Numerator = number of *P. pestis* ingested by 100 neutrophiles.
Denominator = percentage of neutrophiles containing *P. pestis*.
c. HIB = Difco heart infusion broth.
d. *In vivo* = grown in the peritoneal cavity of guinea pigs.
e. FI = Fraction I.

TABLE II. A COMPARISON OF THE PHAGOCYTOSIS RESISTANCE OF SOME VIRULENT AND AVIRULENT STRAINS OF *PASTEURELLA PESTIS* TO GUINEA PIG AND MOUSE PHAGOCYTES[a]

TEST SYSTEM				RESULTS						
P. pestis Culture		*P. pestis* / Leucocyte		Phagocytic Activity				Antigens Detected		
Strain	Cultivation	Mouse	Guinea Pig	Neutrophile		Macrophage		FI	V	W
				Mouse	G.P.	Mouse	G.P.			
Virulent strains:										
Alexander	HIB, 26°C	22.2	9.3	756/93	179/42	336/60	312/92	-	-	-
Alexander	*In vivo*, 38.8°C	26.9	11.3	4/4	6/6	0/0	18/16	+	+	+
Joe	*In vivo*, 38.8°C	22.2	9.3	0/0	8/3	0/0	14/11	+	+	+
M-23	*In vivo*, 38.8°C	27.0	11.3	30/20	12/12	124/32	283/85	-	+	+
M-41	*In vivo*, 38.8°C	23.8	10.0	42/30	29/24	120/30	304/90	-	+	+
Avirulent strains:										
A-1224	*In vivo*, 38.8°C	23.8	10.0	9/3	10/9	3/3	22/15	+	-	-
Control	(uninoculated HIB medium)			0/0	0/0	0/0	0/0	-	-	-

a. Contained in the blood of mice and induced peritoneal exudate of guinea pigs.

IV. DISCUSSION

In the years since Metschnikoff first observed that phagocytic cells of the host were able to destroy invading bacteria, many workers have tended to assume that phagocytosis of a particular pathogen is *prima facie* evidence of a successful defensive action on the part of the host and, therefore, the ability of a pathogen to resist phagocytosis must be an important factor in determining its virulence. However, as Theobald Smith[10] pointed out, Metschnikoff himself accepted limitations to this idea and was aware that some bacteria were able to overcome the phagocytes that ingested them. In considering the pathogenesis of plague, a great deal of importance has been attached to the ability of virulent *P. pestis* strains to acquire resistance to phagocytosis, and the antigens associated with this resistance are thought to be the major determinants of the virulence of these bacilli. In view of our earlier observation that phagocytosis-resistant plague bacilli were readily ingested by fixed macrophages[8] and Cavanaugh's observation that virulent *P. pestis* multiplied within free macrophages,[7] we decided to make a more critical examination of the relationship between the virulence of *P. pestis* and the phagocytic defense system of the host.

Our study reveals that there is no strict correlation between the virulence of *P. pestis* strains and their ability to resist phagocytosis. In fact, some avirulent strains, which are fully toxigenic and capable of growing in the blood of guinea pigs,* acquire a greater degree of resistance to phagocytosis than some virulent strains. This high degree of resistance is associated with the ability to produce the capsular antigen Fraction I, which does not appear to be an essential determinant of virulence. The V and W antigens are apparently essential to the virulence of plague bacilli, but are associated with a much more limited development of resistance to phagocytosis. This evidence and the observations previously mentioned suggest that the major factors determining the virulence of *P. pestis* are those that permit the survival and multiplication of the organisms within phagocytic cells of the host. This may well be the function of the V and W antigens, which are universally found in virulent strains and lacking in most avirulent strains. Further evidence in support of this conclusion has been obtained and will be reported later.

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* To be reported.

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